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| <b>(54) Title:</b> PLANT PARASITIC NEMATODE CONTROL<br><br><b>(57) Abstract</b><br><br>A method of controlling nematodes, the method includes the steps of identification of a gene induced within a successfully infected plant by nematode infection of said plant and modifying the gene to confer nematode resistance to the plant.<br><br><div data-bbox="812 1690 1464 1837" style="border: 1px solid black; padding: 5px; margin-top: 20px;"><b>ATTORNEY DOCKET NUMBER:</b> 9341-027-999<br/><b>SERIAL NUMBER:</b> 09/978,273<br/><b>REFERENCE:</b> AF</div>  |  |  |

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## PLANT PARASITIC NEMATODE CONTROL

This invention relates to control of plant parasitic nematodes, especially to a method of control of root cyst nematodes. This invention also relates to the introduction of nematode resistance into plants of a wide number of species which are susceptible to root cyst nematodes, for example potato plants (Solanum tuberosum)

Cyst nematodes (principally Heterodera and Globodera spp) are key pests of major crops. They are responsible for direct loss in yield and also for indirect losses for instance due to the cost of pesticide and non-optimal use of land of rotation. Potato cyst nematodes (Globodera rostochiensis and Globodera pallida) are key pests of the potato in UK, many other parts of Europe and in other principal potato growing areas. Heterodera glycines (Soybean cyst nematode) has an economic effect on soybean that may exceed \$500m/y in USA alone. Heterodera schachtii (beet cyst nematode) is a major problem for sugar beet growers in Europe and USA. Heterodera avenae (cereal cyst nematode) has a worldwide economic status.

Economically significant densities of cyst nematodes usually cause stunting of crop plants. The root system is smaller than for uninfected plants, resulting in leaves showing symptoms of mineral deficiencies with an increased risk of wilting in dry soil conditions. Yield losses are related to the density of cyst nematode present at planting and in severe cases may be substantially above 50% for crops such as potato and soybean.

Current control depends upon chemicals, cultural techniques and resistant varieties all of which may be used in an integrated manner. Improved control is required. Nematicides are among the most unacceptable crop protection chemicals in widespread use. For instance Aldicarb and

its breakdown products are highly toxic to mammals and have polluted groundwater. The result is that governments of several states within USA have placed restriction on the use of this nematocide. In The Netherlands there is a policy of reduction in nematocide use over a five year period. Cultural control is not an ideal solution to nematode control because measures such as crop rotation include hidden losses that are unacceptable to specialist growers or those with few economic alternative crops.

Resistance of a plant to nematodes may be effected by the inability of the nematode to reproduce on a genotype of a host plant species, and dominant, partially dominant and recessive modes of inheritance occur based on one to several plant genes. However, their commercial value is limited for the plant breeder and farmer. For instance, in potato different sources of resistance occur and create subdivision of populations in Europe, as in the case of the single dominant gene H1 which confers resistance against G.rostochiensis (Ro1 and Ro4) but not other forms of this species (Ro2 Ro3 and Ro5).

The cultivar Maris Piper expresses H1 and is widely used in the UK against G.rostochiensis but its use has been correlated with an increased prevalence of G.pallida which is able to reproduce in the presence of H1. The problem posed by resistance-breaking pathotypes occurs for other cyst nematodes and sources of resistance are known. In some cases, sources of resistance are polygenic, presenting more difficulties for the plant breeder. In addition the resistance in some cultivars is quantitative rather than qualitative in nature and so increased multiplication may occur with time in response to frequent use of such cultivars.

The interface between plant and pathogen is a site of key importance in the determination of susceptibility or resistance to invasion. During early determinative stages of the invasion process, the interface is restricted to a

very small number of plant cells at the local site of infection. Later redifferentiation of existing plant cells forms a syncytium from which the animal feeds. The syncytium is induced by second-stage juveniles after they have migrated into the roots of a host probably in response to secretions released by the animal into an initial feeding cell. When the plant is susceptible, the syncytium increases in volume and is maintained throughout the feeding period of the nematode. In the case of females this may occupy several weeks. The cell biology of the syncytium is well characterised.

This invention seeks to provide a method of conferring nematode resistance in a plant which can be universal in its protection against cyst nematodes and can have long term persistence. The invention contrasts with any method which may involve the use of a known resistance gene being instead based on the use of genes which are specifically part of the susceptibility to nematode attack.

It relates to identification of previously unknown genes expressed specifically within the feeding site of a cyst nematode and subsequent to the attachment by the nematode. Identification of the genes may be achieved through use of a polymerase chain reaction (PCR). Previously the limited cellular interface between plant and pathogen has limited understanding of the molecular biology at the interface. The invention further relates to novel use of such genes to direct, upon attack, the release of materials specifically within the feeding site, which are toxic to either plant cells or nematodes. The specific release of such materials enables compounds to be used, which if expressed constitutively within the plant, would impair plant growth or result in an unacceptable toxicological hazard. A range of such compounds is already known and standard biotechnological techniques are available for their application with the invention.

Corresponding nematode specific genes can be

demonstrated across a wide range of plants and it is possible to protect any crops which are capable of being genetically manipulated to express promoters of feeding site characteristic genes linked to sequences capable of disrupting nematode reproduction. A few illustrative but non-limiting examples include potato, tomato, sugar beet and tobacco.

Syncytium development is also known to be similar for a variety of cyst nematodes. Use of a specifically triggered gene has therefore universal applicability to all cyst nematodes. This includes, but is not limited to: Globodera pallida and Globodera rostochiensis (potato cyst nematodes), Heterodera glycines (soybean cyst nematodes), Heterodera shachtii (beet cyst nematode), Heterodera avenae (cereal cyst nematode), Heterodera oryzae (rice cyst nematode) and Globodera tabacum (tobacco cyst nematode).

In this specification the term "gene" refers to a DNA sequence that incorporates (1) upstream (5') regulatory signals including the promoter, (2) a coding region specifying the product, protein or RNA of the gene and (3) downstream (3') regions including transcription termination and polyadenylation signals (4) associated sequences required for efficient and specific expression.

The term "promoter" refers to a region of a DNA sequence that incorporates the necessary signals for the efficient expression of a coding sequence. This may include sequences to which an RNA polymerase binds but is not limited to such sequences and may include regions to which other regulatory proteins bind, together with regions involved in the control of protein translation and may include coding sequence.

According to the first aspect of the present invention there is provided a method of controlling nematodes, the method including the steps of identification of a gene induced within a successfully infected plant by nematode infection of said plant and modifying the gene to confer

nematode resistance to the plant.

The methods preferred comprise identification of genes induced at the feeding site by nematode infection of a plant said genes being characteristic of nematode infection.

A preferred method of identification of the genes includes the steps of:

constructing a cDNA library by use of a polymerase chain reaction (PCR);

comparing said library with cDNA of infected and uninfected plants; and

identifying a cDNA clone representing a gene expressed at the feeding site and using the cDNA clone to isolate a corresponding genomic clone.

The gene or part thereof may include a DNA sequence consisting essentially of the sequence selected from the group comprising:

GCCCAAACCTTTCCGGTGTACTCCTTGTCCTTGTTTTTTGTAGTCTTTTACCTATCCAAC  
AAAAATTTCTCGCCAAAAAAGGGTTATAACACCGCGATAAAGCTCTTAAATAATG  
(formula 1)

AACATCGGGTCCAAGAGAGGAAAAAGGCACGAAGATGGACAATTTTACCAAAGCATT  
CCTTAGGCTCATAAAGCATTTTAAACCCCGATGCTGTTGTTGTTTTGAAGG  
(formula 2)

GATCCACGCCTCTGAATAGCACAGAAACAGAGTCTACAAGAAAAGCACACATATTTTTG  
CAGTTGGAGAAATAACGAGCCATTGTAATTGNCGGTTCTAAGNNTCGAAGCGATCAAAT  
TAAATTAAAGTTAGCAACGG (formula 3)

CATGACGATGGACAAAATCATTGAGGAACTGGATAACACCGNCGGCTGCCGGGGCTGGC  
GAATCTGTGGGTNCCGCCAATTGTAACCGTATCGATATGCTCTCAACCGGCATTAAAA  
(formula 4)

CAGCATTACACTGGCCCAGGTGCAGTACAGCATGTGGGTGACGNGGAAANANNNCCTGGT  
ACTTTTCGGAACCTATGCACACCGGCTGCTATCAAAGCCTGAAGGCCTGCATA

(formula 5)

GTCCGTACCTTTTCGGGACGCAATACCGTATTGCTGCGCTTCCAGAGAGTCACCTACCGCT  
TTGAATGAC (formula 6)

GCGCCGCGGCACATCGGGGGCTCGGNGGCTACGGCTACGGAGGTTGCACAACTTGCGGAC  
GCCAAATAAACGCGCAACAATCGG (formula 7)

CGTGAGTCAGTNAGTCGTATTACAATTCAGTGGCCGTCGTTTTACAACGTCGTGCTGACT  
GGGAAACCC (formula 8)

wherein A is adenine

C is cytosine

T is thymine

G is guanine

and N is an unassigned nucleotide

The DNA sequence mentioned above or in the following statements of invention may consist essentially of any of the above sequences (formulae 1 to 8) or equivalent nucleotide sequences representing alleles or polymorphic variants of these genes.

According to the second aspect of the present invention a nematode resistant plant incorporates a gene or part thereof, the gene including a DNA sequence consisting essentially of a sequence selected from the group comprising:

GCCCAAACCTTTCCGGTGTACTCCTTGTCTTGTGTTTTGTAGTCTTTTACCTATCCAAC  
AAAAATTTCTCGCCAAAAAAGGGTTATAACACCGCGATAAAGCTCTTAAATAATG  
(formula 1)

AACATCGGGTCCAAGAGAGGAAAAGGCACGAAGAATGGACAATTTTACCAAAGCATT  
CCTTAGGCTCATAAAGCATTTTAAACCCCGATGCTGTTGTTGTTTTGAAGG  
(formula 2)



GTATCCACGCCCTCTGAATAGCACAGAAACAGAGTCTACAAGAAAAGCACACATATTTTGG  
CAGTTGGAGAAATAACGAGCCATTGTAATTGNCGGTTCTAAGNNTCGAAGCGATCAAAAT  
TAAATTAAAGTTAGCAACGG (formula 3)

CATGACGATGGACAAAATCATTGAGGAACTGGATAACACCGNNGGGCTGCCGGGGCTGGC  
GAATCTGTGGGTNCCGCCAATTCGTAACCGTATCGATATGCTCTCAACCGGCATTAAAA  
(formula 4)

CAGCATTACACTGGCCCAGGTGCAGTACAGCATGTGGGTGACGNGGAAANANNNCCTGGT  
ACTTTTCGGAACATATGCACACCGGCTGCTATCAAAGCCTGAAGGCCTGCATA  
(formula 5)

GTCGCTACCTTTCCGGGACGCAATACCGTATTGCTGCGCTTCCAGAGAGTCACCTACCGCT  
TTGAATGAC (formula 6)

GGCCCGCGGCACATCGGGGGCTCGGNGGCTACGGCTACGGAGGTTGCACAACTTGCGGAC  
GCAATAAACGCGCAACAATCGG (formula 7)

CGTGAGTCAGTNAGTCGTATTACAATTCAGTGGCCGTCGTTTTACAACGTCGTCGTGACT  
GGGAAACCC (formula 8)

wherein A is adenine

C is cytosine

T is thymine

G is guanine

and N is an unassigned nucleotide

According to the third aspect of the present invention there is provided use of a plant gene promoter region identified by the method of a preceding aspect of this invention for conferring root cyst nematode resistance to a plant

The method may comprise the steps of modifying or transforming the plant with the promoters to genes of preceding aspect of this invention and further modifying the plant by linkage to a means of killing a plant cell

that attempts re-differentiation towards syncytial development following stimulation by a nematode.

The promoter sequence of a gene clone which is expressed within the feeding site could be used to promote the following substances:

a. A toxic protein, peptide or an enzyme such as DNase, RNase or a proteinase

b. A multigene toxic syndrome, whereby, for example, the promoter may express a protein or peptide which is inactive until activated by a protein produced by a gene coupled to another promoter sequence identified as feeding site specific. The product of a first gene A may be inactive until attacked by the product of the second gene B to form a new A' which represents an active toxin or protease. Alternatively A may be inactive without B such that only A-B molecules are active.

c. Antisense RNA, including one or more antisense RNA genes being the same as the gene sequences of the invention or other feeding site specific genes. That is the promoter may be used to direct the production of its own antisense RNA. This dramatically reduces the level of the normal product. Alternatively the promoter may be used to direct expression of an antisense RNA for general cellular genes, such as an ATP synthetase, essential for cell viability.

Cyst nematode resistance may also be conferred on a plant specifically at the site of feeding, the method comprising the steps of modifying or transforming a plant with the promoters to genes of a preceding aspect of this invention in conjunction with a gene whose product has a direct lethal or pronounced sub-lethal effect on the nematode after ingestion (ie. an anti-nematode gene product). Examples of potentially useful genes that could be used in conjunction with the current invention are:

a. a protein toxin of Bacillus thuringiensis (or a similar organism) having anti-nematode activity.

b. the gene for a nematode toxic prot in in accordance with UK patent application numb r 9104617.7

c. an antibody that disrupts feeding by interacting with the ingestion or digestion of food such as one of the antibodies described for soybean cyst nematode including that against the dorsal pharyngeal gland (Atkinson et al, 1988 Annals of Applied Biology 112, 459-469) using the procedures for transgenic expression of antibodies in plants described by Hiatt, A. Cafferkey, R C. & Bowdish, K. (1989 Production of Antibodies in Transgenic Plants Nature 342, 76 - 78).

d. a protein or peptide, such as a neuropeptide that would have a toxic effect upon the feeding cyst nematode following ingestion.

It will be appreciated that the level of resistance need not be absolute but it will generally be conferred to a degree which is agriculturally or economically significant for that plant.

The invention affords a means of providing nematode resistance that is localised to the site of feeding or adjacent thereto of the nematode. It overcomes the need for the plant to produce constitutively an anti-nematode gene product (such as examples listed in the third aspect of the invention). This limits any detrimental effect on the plant that may arise from constitutive expression. Many crops are protectable from cyst nematodes by this invention and are therefore candidates for being genetically manipulated to express the promoters of the syncytium-characteristic genes linked to sequences that either disrupts the redifferentiation of plant cells to form a syncytium or expresses an anti-nematode gene product within the syncytium. These include the crops attacked by cyst nematodes such as Globodera pallida and Globodera rostochiensis (potato cyst nematodes), Heterodera glycines (soybean cyst nematode), Heterodera shachtii (beet cyst nematode), Heterodera avenae (cereal cyst nematode),

Heterodera carotae (carrot cyst nematode), Heterodera oryzae (rice cyst nematode) and Globodera tabacum (tobacco cyst nematode). A few illustrative but non-limiting examples of crops which may be protected by this invention from cyst nematodes include potato, tomato, soybean, sugar beet, oilseed rape, wheat, oats, barley, rice, carrot, brassicas and tobacco.

The invention is further described by means of example but not in any limitative sense.

The first example describes the methods used to identify, characterise and manipulate potato genes to generate resistant transgenic potato plants.

#### Example 1

This example illustrates the use of a susceptible cultivar of potato to Globodera rostochiensis pathotype Ro2 to allow identification of genes expressed within the feeding site of the nematode. Sections 1 to 5 provide details for the production of infected root tissue. The minute amounts of locally infected tissue provided quantities of RNA (Section 6) best suited to construction of a cDNA library by a PCR amplification approach (Section 7.1 to 7.4). Isolation of feeding cell specific cDNA clones was achieved by differential hybridisation screening with cDNA probes from infected and non-infected tissue (Section 7.5). Positive cDNA clones were analysed by DNA sequencing (Section 10) and the inserts used as probes in subsequent steps. An examination of temporal and spatial expression of genes was performed by Northern analysis (Section 8) using a range of plant tissues and time-course of infection. Further localisation of gene expression was achieved by in situ hybridisation studies (Section 12). Genomic clones corresponding to feeding cell specific cDNA clones were isolated from a genomic DNA library (Section 11). Promoters were identified by DNA sequence analysis

(Section 11) for the development of constructs suitable for generating transgenic potato plants expressing nematode resistance (Section 13) locally within the feeding site.

### 1. Plant Materials

Solanum tuberosum cultivar Maris Piper (Scottish seed potatoes, class A) were chitted up for 24 days (Hammond-Kosack K E, Atkinson H J, Bowles D J 1990 Changes in abundance of translatable mRNA species in potato roots and leaves following root invasion by cyst-nematode G. rostochiensis pathotypes *Physiol Mol Plant Pathol*). Individual sprouts were excised from growth in pouches or whole tubers were used for pot grown material.

### 2. Nematodes

Population of Globodera rostochiensis pathotype Ro1 and Ro2 cysts were maintained as dry stocks at 4°C. The cysts were soaked in tap water for 7 days at 4°C. Potato root diffusate was added (Shepherd A M 1986 In: Southey J F (ed) Laboratory methods for work with plant and soil nematodes: MAFF reference book 402, 6th edn) and hatching occurred after 4-7 days at room temperature.

### 3. Infection

Pouch material: Individual sprouts were supported in ready-made pouches (Northup-King, Minnesota). 1 cm x 0.5 cm strips of Whatman GFA paper were placed under individual root tips. 50 worms in 10 ul of root diffusate were pipetted onto each root tip which were then overlaid with a second strip of GFA. The GFA filter paper was removed 24 hours after inoculation to synchronize infections. The base was excised and pouches were irrigated in tap water.

Pot material: chitted tubers were planted in 25 cm

pots in soil plus sufficient number of unhatched cysts to give a population of 50 eggs per gram soil.

Sham-inoculated pouches and pots were set up under identical conditions but without cysts.

#### 4. Growth conditions

The infected potatoes were grown under a regime of 18 hours light at 22°C, and 6 hours dark at 18°C

#### 5. Harvesting and dissection

Pouch grown tissue was harvested 4, 7, 15, and 24 days post invasion. The discoloured locally infected roots were dissected directly into foil envelopes in liquid nitrogen. Root tissue directly adjacent the infected area was also dissected and frozen separately.

Pot grown tissue was harvested 26 days post Ro2 invasion. The emerging females and cysts were removed with a fine paint brush and locally infected tissue was dissected directly into liquid nitrogen in individual foil packets.

#### 6. Isolation of total RNA

Foil packets were gently crushed with a pestle to give a powder of tissue that was stored at -70°C until required. Powdered tissue (12mg) was transferred to pre-cooled 20 ml disposable plastic Sarstedt tubes in liquid nitrogen before addition of 0.5 ml phenol/CH<sub>3</sub>Cl/isoamyl alcohol (25:24:1) and 0.5 ml GuHCl buffer (8M Guanidinium-HCl, 20 mM MES (4-morpholino-ethanol-sulphonic acid), 20 mM Na<sub>2</sub>EDTA, adjusted to pH7 with NaOH and 2-mercaptoethanol added to 50 mM, immediately prior to use). Once the tubes had thawed on ice the tissue was homogenized by 15 strokes of a Polytron blender (Kinematica, Switzerland). The aqueous phase was

re-extracted three more times with phenol before overnight precipitation at  $-20^{\circ}\text{C}$  following the addition of 0.2 vol 1M acetic acid and 0.7 vol cold 96% ethanol. RNA recovered by centrifugation at approximately 13,000 xg for 15 minutes in a microcentrifuge was washed in 400 ul 3 M sodium acetate (pH 5.5) at  $4^{\circ}\text{C}$  then in 70% cold ethanol before being finally dissolved in 30-50 ul DEPC-treated  $\text{H}_2\text{O}$  and stored at  $-70^{\circ}\text{C}$ .

## 7. Construction of a PCR-based cDNA library

### 7.1 cDNA synthesis

1 microgram total RNA from potato roots collected 26 days post infection with *G. rostochiensis* pathotype Ro2 was denatured for 5 minutes in a  $70^{\circ}\text{C}$  water bath then rapidly cooled on ice. This sample was added to a reaction mix consisting of 4 ul 5 x AMVRT buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50 mM  $\text{MgCl}_2$ , 5 mM DDT, 5 mM EDTA, 50 ug/ml bovine serum albumin), 2 ul Oligo ( $\text{dT}_{17}$ ) (100 ug/ml), 2 ul dNTP mix (10 mM each ATP, dCTP, dGTP, dTTP), 1 ul 10 mM spermidine-HCl, 1 ul 80 mM sodium pyrophosphate and 125 units human placental ribonuclease inhibitor (HPRNI). 10 units of avian myeloblastoma virus (AMV) reverse transcriptase were added to give a final reaction volume of 20 ul before incubation for 1 hour at  $42^{\circ}\text{C}$ . The reaction was terminated by addition of 20 ul 0.1 M NaCl, 40 mM EDTA. The length and quality of the products were assessed by setting up a parallel reaction containing 50 uCi of  $^{32}\text{P}$ -labelled dCTP (3000 Ci/mmol). The labelled reaction products were fractionated through an alkaline gel that was subsequently autoradiographed. The bulk of labelled products fell within the 200 to 2000 bp size range, with larger products clearly visible.

The oligo dT primer was removed by selective precipitation. 0.5 ug poly I. poly C (Pharmacia) and 3 ul 10% (w/v) CTAB were added to the reaction mix before

centrifugation in a microcentrifuge at room temperature for 20 minutes. The supernatant was carefully removed and the pellet resuspended in 14  $\mu$ l 1M NaCl to which 25  $\mu$ l  $H_2O$  and 1  $\mu$ l 10% CTAB were added and the sample was again pelleted in a microcentrifuge. The pellet was finally resuspended in 10  $\mu$ l 1M NaCl and precipitated in 2.7 vol ethanol overnight at  $-20^{\circ}C$ . The pellet was washed in 70% ethanol and dissolved in 7  $\mu$ l  $H_2O$ . A dG tailing reaction was performed by adding to this sample: 4  $\mu$ l 5 x tailing buffer (1M potassium cacodylate, 125 mM Tris HCl, pH 7.2), 1  $\mu$ l 2 mM DTT, 2  $\mu$ l 5 mM cobalt chloride, 5  $\mu$ l 20 M dGTP and 25 units Terminal transferase (Boehringer) and incubating at  $37^{\circ}C$  for 20 minutes before terminating with 4  $\mu$ l 100 mM EDTA and 2  $\mu$ l 1M NaCl. Excess nucleotides were removed by adding 1  $\mu$ l 10% (w/v) CTAB and spinning in a microcentrifuge at  $4^{\circ}C$  for 20 minutes. The pellet was dissolved in 10  $\mu$ l 1M NaCl before addition of 0.25  $\mu$ g glycogen and 30  $\mu$ l of cold ethanol and reprecipitation overnight at  $-20^{\circ}C$ . After washing the pellet in 70% ethanol it was dissolved in 20  $\mu$ l 50 mM NaOH, 2 mM EDTA and incubated at  $65^{\circ}C$  for 60 minutes to hydrolyse the RNA. The cDNA was again ethanol precipitated, washed and the pellet dissolved in 20  $\mu$ l  $H_2O$ .

## 7.2 PCR Amplification

PCR amplification of homopolymer tailed first strand cDNA utilized the amplimers;

\* A (oligo dT<sub>17</sub>-Not I); GCGGCCGCTTTTTTTTTTTTTTTT

\* B (oligo dC<sub>14</sub>-Eco RI); AAGGAATCCCCCCCCCCCCC

The PCR reactions contained 10  $\mu$ l 10 x PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, 40 mM MgCl<sub>2</sub>, 0.1% gelatin), 10  $\mu$ l 10 x dNTP mix (5 mM each dATP, dCTP, dGTP, dTTP), 100 pmoles amplimer A, 100 pmoles amplimer B, 2 units Taq polymerase and 1-10 ng oligo (dG) tailed cDNA in a total reaction volume of 20  $\mu$ l overlaid with 20  $\mu$ l



mineral oil. The following thermal cycling regime was then performed: 1 cycle 96°C, 2 minutes to denature the products; (96°C, 2 minutes, 58°C, 2 minutes, 72°C, 3 minutes) to allow second strand synthesis, 1 cycle (96°C, 2 minutes, 40°C, 2 minutes, 72°C, 2 minutes) to ensure good priming from the oligo (dT) end; 15 cycles (96°C, 2 min; 58°C, 2 min; 72°C, 3 min) to amplify the products. The reaction mix was fractionated by electrophoresis through a NuSieve GTG LMP (FMC Bioproducts) gel using 123 bp DNA ladder as size marker and regions of the gel corresponding to size classes of <500, 500 to 1500, 1500 to 2000 and > 2000 bp were excised and recovered from the gel by freezing at -20°C then centrifuging in a microfuge for 10 min in Spin-X filters (Costar). The PCR products were Southern blotted and hybridized to 3 clones known to be constitutively expressed in potato roots. cDNA probes of long and short and high and low abundance transcripts hybridized to PCR products reflecting a fair representation of different transcripts with the library.

### 7.3 Library Construction

The recovered DNA (greater than 200 bp) was digested with EcoRI and NotI and ligated into similarly digested lambda-ZAP before packaging in vitro using Stratagene Giga Plus packaging mix according to the manufacturers instructions. The library was titred on E.coli strain XL1-Blue using LB plates with 1% top agar containing 12.5 mM IPTG and 6.25 mg/ml X-gal.

### 7.4 Assessment of library

The quality of the PCR based on CDNA library was assessed in two ways, (i) approximately 96% of plaques proved to be recombinants as shown by insertional inactivation of beta-galactosidase alpha-complementation.

(ii) Plaques picked at random into PCR buffer and amplified using complementary M13 amplimers showed single inserts ranging from 230 bp-1000 bp with an average insert size of 470 bp.

### 7.5 Differential Screening

The library yielded  $3.5 \times 10^5$  recombinant phage of which  $6 \times 10^4$  were screened with  $^{32}\text{P}$  dCTP-labelled probes synthesized as first strand cDNA from 10  $\mu\text{l}$  total RNA prepared from either infected root tissue 26 days post Ro2 invasion or from healthy roots of equivalent age (Curr S J, McPherson M J 1991 PCR-based cDNA library construction. In: McPherson M J, Quirke P, Taylor G R (eds) Polymerase chain reaction: A practical approach. IRL Press at Oxford University Press, Oxford). Unincorporated isotope was removed by precipitation with ammonium acetate and the probes adjusted to give equivalent cpm/ $\mu\text{g}$  cDNA/ml hybridization fluid following liquid scintillation counting. Plaque lifts were performed in duplicate and were denatured in 0.5 M NaOH, 1.5 M NaCl and neutralized in 1.5, 0.5 M Tris pH7.2 and 1 mM EDTA and baked ( $80^\circ\text{C}$ , 2 hours). Filters were prehybridized in separate "healthy" and "infected" prehybridization fluid containing 5 X SSPE, 6% PEG 6000, 0.5% Marvel (skimmed milk powder) 1% SDS, 0.1% sodium pyrophosphate and 250  $\mu\text{g}/\text{ml}$  ultrasonicated and denatured herring sperm DNA at  $65^\circ\text{C}$  for 6 hours. Hybridization was at  $65^\circ\text{C}$  for 24 hours following addition of the appropriate radiolabelled 1st strand probe. Following hybridization the filters were washed sequentially in 5 x SSC, 0.1% SDS then 1 x SSC, 0.1% SDS at  $65^\circ\text{C}$  for 2 x 20 min changes in each solution. The blots were exposed to X-ray film, with intensifier screens at  $-70^\circ\text{C}$ . Plaques which hybridized to Ro 2 day 26 probe and not to healthy day 26 probe were purified to homogeneity by 2 further rounds of plaque screening.

### 8. Northern and Southern hybridization and probe preparation

RNA loaded at 10 ug per track was separated through 0.6M formaldehyde agarose gels running at  $7.5 \text{ Vcm}^{-1}$ . The gel was photographed and rinsed 2 x 20 min in 10 x SSC and blotted onto Hybond-N membrane (Amersham) with 10 x SSC. Filters were baked (80°C, 2hr) and prehybridized for 12 hours at 42°C in 50% formamide, 5 x SSC, 1 x Denhardt's, 100 ug/ml herring sperm DNA and 250 mM phosphate buffer, (pH 6.5). Hybridization was performed in fresh prehybridization buffer including heat denatured  $^{32}\text{P}$ -hexaprime-labelled pPSR 2-4 probe (25 ng) (Feinberg, A P & Vogelstein, B 1983 Nucleic Acids Research 14, 2229 at 42°C for 24 hours. The filters were washed sequentially in 5 x SSC 0.1% SDS at 42°C then 2 x SSC 0.1% SDS at 42°C with a final wash at 65°C in 2 x SSC 0.1% SDS. The blots were exposed to X-ray film, with intensifier screens, at -70°C. PMR1 insert was prepared by picking a single purified plaque into PCR buffer and amplified with complementary M13 amplimers according to Gussow, D & Clackson, T (1989 Nucleic Acids Research 17, 4000).

Genomic DNA was prepared from nuclei isolated from young potato leaves (cultivar Maris Piper) as described by Jofuku, K D & Goldberg, R B (1989, In Plant Molecular Biology: A Practical Approach (ed C S Shaw) pp 37 - 66). The high molecular weight DNA was size fractionated through a 0.6% agarose gel following digestion with the restriction enzymes. To analyse gene expression throughout the plant, RNA was extracted from stems, petioles, leaves, flowers and roots from both infected and uninfected. To analyse the wound response, plant roots or leaves were crushed and RNA extracted from treated tissue over a time-course (3, 6, 9 and 12 hours post crushing). Samples were compared by Northern analysis with RNA extracted from nematode infected

roots. Gels were depurinated, d natured and blotted onto Hybond-N membrane (Sambrook, J. Fritsch, E F & Maniatis, T (1989 Molecular Cloning: A laboratory manual 2nd edn. Cold Spring Harbour Press). The filter was baked (80°C, 2 hr) and prehybridized for 6 hours at 65°C in prehybridization fluid as described for differential screening of plaques. Homogenous hybridization and washing conditions were as described for northern blot filters. Heterologous hybridizations were performed at 56°C and fillers were washed in 5 x SSC at 56°C.

#### 9. Excision of pBluescript from lambda ZAP and plasmid purification

pBluescript clone PMR1, carrying the insert whose sequence is shown in one of formulae 1 to 8, was excised from a positively hybridizing lambda ZAP clone and rescued according to the instructions in the Stratagene manual. Plasmid DNA was purified by caesium chloride density gradient centrifugation (Davis L G, Dibner M D, Batey J F 1986 Basic methods in molecular biology Elsevier Science Publishers, New York)

#### 10. Sequence analysis

DNA sequence analysis was performed on alkaline denatured plasmid DNA according to the methods described in the manufacturers protocols for Sequenase (Version 2.0) reactions (United States Biochemical Corp). DNA sequence data were compared with the EMBL data base and GenBank database (release 23) by using the QUICKN program that uses the algorithm of Lipman and Pearson (Lipman D J K, Pearson W R 1985 Rapid and sensitive protein similarity searches Science 227: 1435-1441) running on a VAX 11/750 computer VMS operating system.

### 11. Isolation of genomic clone

A lambda EMBL3 genomic DNA library was constructed using partial EcoRI digested cv Maris Piper genomic DNA according to methods described by Sambrook et al (1989) ibid. The library was screened by plating approx  $1.8 \times 10^5$  clones, preparing duplicate plaque lift filters, prehybridising then hybridising and washing essentially as previously described under Section 7.5 except that the probe was prepared by hexa-prime labelling insert DNA. PCR amplified (Gussow D, Clackson T 1989 Direct clone characterization from plaques and colonies by the polymerase chain reaction Nucleic Acids Res 17:10) from the cDNA clones, pPMR1, pPMR2 or pPMR3.

Positively hybridising clones recovered after 3 rounds of hybridisation screening were further characterised by restriction analysis and DNA sequencing Sambrook et al (1989 ibid) to localise promoter sequences.

### 12. In situ hybridisations

Tissue from infected and uninfected roots were embedded in wax, sectioned, prehybridised then hybridised to sense and antisense probes of pPMR1, pPMR2 and pPMR3 as described by Jackson D. (In Molecular Plant Pathology: A Practical Approach (ed. S J Gurr, M J McPherson and D J Bowles IRL Press, Oxford, 1991)).

### 13. Production and regeneration of transgenic plants

Transgenic potatoes were produced according to the methods described Harsh et al (1985 Science 227, 1229 - 1231) using the Agrobacterium strain GV3101 containing pGV3850, carrying feeding cell specific promoters fused to an appropriate disruptive gene selected from those mentioned in the introduction to this specification, and

plantlets were regenerated prior to testing for nematode resistance.

#### Example 2

##### Presence of equivalent genes in other plant species

Genes from one species of plant can be expressed in a temporally and spatially correct manner in a second species even when the species belong to different plant families. Due to the similarity in physiology and pathology of syncytium establishment and life cycles of cyst nematodes on a range of plants from many families, a gene expressed within the syncytium of one plant will be correctly expressed upon introduction to a different plant species.

Confirmation is obtained by identifying equivalent genes within other species of plants. Tobacco, tomato and sugar beet genomic DNA digests were examined by hybridisation to pPMR1 (following the method of Section 8). In all cases cross hybridization was observed showing that equivalent genes exist in these plants. A construct comprising the promoter of the gene represented by pPMR1 is therefore capable of directing the correct localised expression of a suitable anti-nematode gene in a range of crop plants. These experiments also provide a route to isolation of equivalent gene from other species of plant for the expression of an anti nematode defence mechanism therein.

## CLAIMS

1. A method of controlling nematodes, the method including the steps of identification of a gene induced within a successfully infected plant by nematode infection of said plant and modifying the gene to confer nematode resistance to the plant.

2. A method of controlling nematodes as claimed in claim 1, including the step of identification genes characteristic of nematode infection induced by nematode infection at the feeding site within in plant

3. A method of controlling nematodes as claimed in claim 1 or claim 2, including the steps of:

constructing a cDNA library by use of a polymerase chain reaction (PCR);

comparing said library with cDNA of infected and uninfected plants; and

identifying a cDNA clone representing a gene expressed at the feeding site and using the cDNA clone to isolate a corresponding genomic clone.

4. A method of controlling nematodes as claimed in any preceding claim, wherein said gene or part thereof includes a DNA sequence consisting essentially of the sequence selected from the group comprising:

GCCCAAACCTTTCCGGTGTACTCCTTGTCCTTGTTTTTGTAGTCTTTTACCTATCCAAC  
AAAAATTTCTCGCCAAAAAAGGGTTATAACACCGCGATAAAGCTCTTAAATAATG  
(formula 1)

AACATCGGGTCCAAGAGAGGAAAAGGCACGAAGAATGGACAATTTTACCAAAGCATT  
CCTTAGGCTCATAAAGCATTTTAAACCCCGATGCTGTTGTTGTTTGAAGG  
(formula 2)

GTATCCACGCCTCTGAATAGCACAGAAACAGAGTCTACAAGAAAAGCACACATATTTTGG  
CAGTTGGAGAAATAACGAGCCATTGTAATTGNCGGTTCTAAGNNTCGAAGCGATCAAAAT  
TAAATTAAAGTTAGCAACGG (formula 3)

CATGACGATGGACAAAATCATTGAGGAACTGGATAACACCGNCGGCTGCCGGGGCTGGC  
GAATCTGTGGGTNCCGCCAATTCGTAACCGTATCGATATGCTCTCAACCGGCATTA  
(formula 4)

CAGCATTACACTGGCCCAGGTGCAGTACAGCATGTGGGTGACGNGGAAANANNNCCTGGT  
ACTTTTCGGAACATATGCACACCGGCTGCTATCAAAGCCTGAAGGCCTGCATA  
(formula 5)

GTCGCTACCTTTTCGGGACGCAATACCGTATTGCTGCGCTTCCAGAGAGTCACCTACCGCT  
TTGAATGAC (formula 6)

GGCCCGCGGCACATCGGGGGCTCGGNGGCTACGGCTACGGAGGTTGCACAACCTGCGGAC  
GCAATAAACGCGCAACAATCGG (formula 7)

CGTGAGTCAGTNAGTCGTATTACAATTCAGTGGCCGTCGTTTTACAACGTCGTCGTGACT  
GGGAAACCC (formula 8)

wherein A is adenine

C is cytosine

T is thymine

G is guanine

and N is an unassigned nucleotide

5. A method of controlling nematodes including the step of combining a promoter region derived from a gene obtained by a method of any preceding claim with further regions coding for products disruptive of nematode attack.

6. A method of controlling nematodes as claimed in claim 5 further including the step of combining the promoter region of a said gene with a region coding for means adapted to kill a cell which attempts redifferentiation towards feeding site development.

7. A method of controlling nematodes as claimed in claim 6 wherein said means adapted to kill a cell are selected from the group comprising:



- i. DNAase, RNAase or proteinase, toxic protein or toxic peptide
- ii. A multi-gene toxic syndrome
- iii. Antisense RNA to the redifferentiated cells
8. A method of controlling nematodes as claimed in claim 5 wherein said further regions code for a product having a lethal or pronounced sublethal effect on ingestion by a nematode.
9. A method of controlling nematodes as claimed in claim 8 wherein said product is selected from the group comprising:
  - i. a nematode toxic protein
  - ii. an antibody disruptive of nematode feeding
  - iii. a nematode toxic neuropeptide
10. A nematode resistant plant incorporating a gene or modified gene derived from a method as claimed in any preceding claim.
11. A method of controlling nematodes as claimed in any preceding claim wherein the nematodes are root cyst nematodes.
12. A method of controlling nematodes as claimed in any preceding claim wherein the plants are selected from the genus Solanum.
13. A method of controlling nematodes as claimed in claim 12 wherein the plant is Solanum tuberosum.
14. A nematode resistant plant incorporating a modified gene created from a gene including a DNA sequence consisting essentially of a sequence selected from the group comprising:

GCCCAAACCTTCCGGTGTACTCCTTGTCCTTGTTTTTGTAGTCTTTTACCTATCCAAC  
AAAAATTTCTCGCCAAAAAAGGGTTATAACACCGCGATAAAGCTCTTAAATAATG  
(formula 1)

AACATCGGGTCCAAGAGAGGAAAAAGCCACGAAGAATGGACAATTTTACCAAAGCATT  
CCTTAGGCTCATAAAGCATTTTAAACCCCGATGCTGTTGTTTGAAGG

(formula 2)

GTATCCACGCCTCTGAATAGCACAGAAACAGAGTCTACAAGAAAAGCACACATATTTTGG  
CAGTTGGAGAAAATAACGAGCCATTGTAATTGNCGGTTCTAAGNNTCGAAGCGATCAAAAT  
TAAATTAAAGTTAGCAACGG (formula 3)

CATGACGATGGACAAAATCATTGAGGAAGTGGATAACACCGNCGGCTGCCGGGGCTGGC  
GAATCTGTGGGTNCCGCCAATTCGTAACCGTATCGATATGCTCTCAACCGGCATTAAAA  
(formula 4)

CAGCATTACACTGGCCCAGGTGCAGTACAGCATGTGGGTGACGNGGAAANANNNCCTGGT  
ACTTTTCGGAAGTATGCACACCGGCTGCTATCAAAGCCTGAAGGCCTGCATA  
(formula 5)

GTCGCTACCTTTTCGGGACGCAATACCGTATTGCTGCGCTTCCAGAGAGTCACCTACCGCT  
TTGAATGAC (formula 6)

GGCCCGGGCACATCGGGGGCTCGGNGGCTACGGCTACGGAGGTTGCACAACTTGCGGAC  
GCAATAAACCGCGCAACAATCGG (formula 7)

CGTGAGTCAGTNAGTCGTATTACAATTCAGTGGCCGTCGTTTTACAACGTCGTCGTGACT  
GGGAAACCC (formula 8)

wherein A is adenine

C is cytosine

T is thymine

G is guanine

and N is an unassigned nucleotide

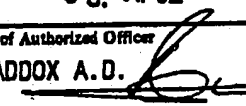
15. A nematode resistant plant of the genus *Solanum* incorporating a gene sequence as claimed in claim 14.

16. Use of a plant gene promoter region identified by the method of any of claims 1 to 13 or in accordance with claim 14 for conferring root cyst nematode resistance to a plant.

## INTERNATIONAL SEARCH REPORT

PCT/GB 91/01540

International Application No.

|  |  |                                     |
|--|--|-------------------------------------|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>  |  |                                     |
| According to International Patent Classification (IPC) or to both National Classification and IPC  |  |                                     |
| Int.Cl. 5 C12N15/82; C12N15/29; A01N65/00; A01H5/00  |  |                                     |
| <b>II. FIELDS SEARCHED</b>   |  |                                     |
| Minimum Documentation Searched <sup>7</sup>  |  |                                     |
| Classification System  | Classification Symbols   |                                     |
| Int.Cl. 5  | C12N ; A01N ; A01H   |                                     |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>   |  |                                     |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>  |  |                                     |
| Category <sup>10</sup>   | Citation of Document <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>  | Relevant to Claim No. <sup>13</sup> |
| A  | J. CELL. BIOCHEM. SUPPL.<br>vol. 12C, 1988, MEETING MARCH 26 - APRIL 1 1988<br>page 266;<br>HAMMOND, H. E., ET. AL.: 'The molecular basis of plant resistance to potato cyst nematode'<br>see the abstract Y217<br>--- | 1-16                                |
| A  | J. CELL. BIOCHEM. SUPPL.<br>vol. 13D, 1989, MEETING APRIL 1-7 1989<br>page 323;<br>NIEBEL, A., ET. AL.: 'Molecular analysis of nematode induced giant cells in potato roots'<br>see the abstract M429<br>---           | 1-16                                |
| A  | EP, A, 0 285 361 (PLANT CELL RESEARCH INST TUTE) 5<br>October 1988<br>see page 4, line 46 - line 53<br>---   | 1-16                                |
| <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> |  |                                     |
| <b>IV. CERTIFICATION</b>   |  |                                     |
| Date of the Actual Completion of the International Search  | Date of Mailing of this International Search Report  |                                     |
| 2 17 DECEMBER 1991   | 03. 01. 92   |                                     |
| International Searching Authority  | Signature of Authorized Officer  |                                     |
| EUR PEAN PATENT OFFICE   | MADDOX A.D.    |                                     |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |   |                       |
|--|---|-----------------------|
| Category *   | Citation of Document, with indication, where appropriate, of the relevant passages  | Relevant to Claim No. |
| A  | <p>BIOLOGICAL ABSTRACTS<br/>vol. 91, 1991, ABSTRACT 100296<br/>&amp; PHYSIOL. MOL. PLANT PATHOL.<br/>vol. 37, no. 5, 1990,<br/>pages 339 - 354;<br/>HAMMOND-KOSACK, K. E., ET AL.,: 'Changes in<br/>abundance of translatable messenger RNA species<br/>in potato roots and leaves following root<br/>invasion by cyst nematode Globodera<br/>rostochiensis pathotypes'<br/>see the abstract</p> <p>---</p> | 1-16                  |
| A  | <p>J. CELL. BIOCHEM. SUPPL.<br/>vol. 15A, 1991, MEETING HELD JAN. 1991<br/>page 56;<br/>GURR, S. J., ET AL.,: 'Identification of plant<br/>genes expressed at the feeding site of the<br/>potato cyst nematode'<br/>see the abstract A214</p> <p>---</p>  | 1-16                  |
| P,A  | <p>MOLECULAR &amp; GENERAL GENETICS<br/>vol. 226, May 1991,<br/>pages 361 - 366;<br/>GURR, S. J., ET AL.,: 'Gene expression in nematode<br/>infected plant roots'<br/>see the whole document</p> <p>---</p>   | 1-16                  |
| A  | <p>BIOLOGICAL ABSTRACTS BR41:121119<br/>&amp; PHYTOPHYLACTICA<br/>vol. 23, no. 2, 1991, SYMPOSIUM HELD APRIL 7-10,<br/>1991.<br/>page 182;<br/>DE WAELE, D.: 'Potential of plant genetic<br/>engineering for nematode control'<br/>see the abstract</p> <p>---</p>  | 1-16                  |

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on  
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